HCR™ RNA flow cytometry (v3.0) protocol for mammalian cells in suspension

This protocol has not been validated for all mammalian cell types and should only be used as a template.

Technical Support
support@molecularinstruments.com

Safety Data Sheets (SDS)
www.molecularinstruments.com/safety-v3

Patents
www.molecularinstruments.com/patents

Ordering for Multiplex Experiment
Order one HCR™ RNA-FISH (v3.0) kit per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH (v3.0) kit for target mRNA1
  - HCR™ Probe (v3.0): target mRNA1 for use with amplifier B1
  - HCR™ Amplifier (v3.0): B1-647
  - HCR™ RNA-FISH Buffers (v3.0): HCR™ Probe Hybridization Buffer (v3.0), HCR™ Probe Wash Buffer (v3.0), HCR™ Amplifier Buffer (v3.0) (for use with all kits)
- HCR™ RNA-FISH (v3.0) kit for target mRNA2
  - HCR™ Probe (v3.0): target mRNA2 for use with amplifier B2
  - HCR™ Amplifier (v3.0): B2-488

Storage conditions

- Store HCR™ Probes (v3.0), HCR™ Amplifiers (v3.0), HCR™ Probe Hybridization Buffer (v3.0), and HCR™ Probe Wash Buffer (v3.0) at -20 °C.
- Store HCR™ Amplifier Buffer (v3.0) at 4 °C.
- On the bench top, keep stock solutions on ice.
- Make sure all solutions are well mixed before use.

Sample preparation protocol

1. Aspirate growth media from culture plate and wash cells with DPBS.
   NOTE: *avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
2. Add 3 mL of trypsin per 10 cm plate and incubate in a 5% CO_2 incubator at 37 °C for 5 min.
3. Quench trypsin by adding 3 mL of growth media.
4. Transfer cells to a 15 mL conical tube and centrifuge for 5 min at 180 × g.
5. Aspirate supernatant and re-suspend cells in 4% formaldehyde to reach approximately 10^6 cells/mL.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
6. Fix cells for at least 1 hr at room temperature.
7. Centrifuge for 5 min at 180 × g and aspirate supernatant.
8. Wash cells 4 times with PBST (use the same volume as formaldehyde solution).
9. Re-suspend cells in cold 70% ethanol (use the same volume as formaldehyde and PBST solutions).
10. Store cells at 4 °C overnight before use.

Multiplexed HCR™ RNA flow cytometry (v3.0) protocol

Detection stage

1. Transfer desired amount (0.5-1 $\times$ 10^6) of fixed cells into a 1.5 mL tube.
2. Centrifuge for 5 min and remove supernatant.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at 180 $\times$ g.*
3. Wash cells twice with 500 μL of PBST. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400 μL of HCR™ Probe Hybridization Buffer (v3.0) and pre-hybridize for 30 min at 37 °C.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*
5. In the meantime, prepare probe solution by adding 2 pmol of each HCR™ Probe (v3.0) (e.g. 2 μL of 1 μM stock) to 100 μL of HCR™ Probe Hybridization Buffer (v3.0) pre-heated to 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
   NOTE: *use higher probe concentration (e.g., 16 nM) to increase signal for quantitative RNA flow cytometry.*
7. Incubate the sample overnight (>12 h) at 37 °C.
8. Centrifuge for 5 min to remove probe solution.
9. Re-suspend the cell pellet with 500 μL of HCR™ Probe Wash Buffer (v3.0).
   CAUTION: *HCR™ Probe Wash Buffer (v3.0) contains formamide, a hazardous material.*
   NOTE: *HCR™ Probe Wash Buffer (v3.0) should be pre-heated to 37°C before use.*
10. Incubate for 10 min at 37 °C and remove the wash solution by centrifugation for 5 min.
11. Repeat steps 9 and 10 for three additional times.
12. Re-suspend the cell pellet with 500 μL of 5$\times$ SSCT.
13. Incubate for 5 min at room temperature.
14. Proceed to hairpin amplification.

Amplification stage

1. Centrifuge for 5 min to pellet the cells.
2. Re-suspend the cell pellet with 150 μL of HCR™ Amplifier Buffer (v3.0) and pre-amplify for 30 min at room temperature.
3. Separately prepare 15 pmol of hairpin h1 and 15 pmol of hairpin h2 by snap cooling 5 μL of 3 μM stock (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
   NOTE: *Hairpins h1 and h2 are provided in hairpin storage buffer ready for snap cooling. h1 and h2 should be snap cooled in separate tubes.*
4. Prepare hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL of HCR™ Amplifier Buffer (v3.0) at room temperature.
5. Add the hairpin solution directly to the sample to reach a final hairpin concentration of 60 nM.
6. Incubate the sample overnight (>12 h) in the dark at room temperature.
7. Centrifuge for 5 min and remove the hairpin solution.
8. Re-suspend the cell pellet with 500 μL of 5× SSCT.
9. Without incubation, remove the wash solution by centrifugation for 5 min.
10. Repeat steps 8 and 9 for five additional times.
11. Re-suspend the cell pellet in desired buffer and volume.
12. Filter cells before flow cytometry.

Buffer recipes

4% formaldehyde in PBST	For 10 mL of solution
4% formaldehyde	2.5 mL of 16% formaldehyde
1× PBS	1 mL of 10× PBS
0.1% Tween 20	100 μL of 10% Tween 20
	Fill up to 10 mL with ultrapure H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O

NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

S1 HCR™ Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR™ RNA-ISH**
  HCR™ RNA in situ hybridization (RNA-ISH) offers unmatched performance, robustness, and versatility imaging RNA targets in diverse organisms and sample types (Choi et al., 2010, Choi et al., 2014, Choi et al., 2018):
  - **HCR™ RNA-FISH**
    HCR™ RNA-FISH enables 10-plex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, quantitative precision, single-molecule fidelity) and ease-of-use (no probe set optimization for new targets and organisms).
  - **Enzymatic HCR™ RNA-CISH/RNA-FISH**
    Enzymatic HCR™ RNA-ISH integrates enzymatic signal amplification to enable extreme-sensitivity RNA imaging using either chromogenic or fluorescent staining (RNA-CISH/RNA-FISH). In tissue sections, entirely protease-free workflows preserve sample morphology and maintain protein target integrity, enabling seamless compatibility with existing immunohistochemistry (IHC)/immunofluorescence (IF) assays. HCR™ RNA-CISH offers the convenience of brightfield microscopy and the option of archival staining.
- **10-Plex HCR™ Spectral Imaging**
  HCR™ RNA-FISH/IF enables quantitative high-resolution imaging of 10 RNA and/or protein targets with 1-step HCR™ signal amplification for all targets simultaneously. The method is suitable even for whole-mounts and delicate samples as it requires no repeated staining, imaging, registration, or stripping (Schulte et al., 2024).
- **HCR™ RNA-FISH/IF**
  HCR™ RNA-FISH/IF enables a unified approach to multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf et al., 2021).
- **HCR™ IF**
  HCR™ IF enables multiplex, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).
- **Subcellular Quantitative RNA and Protein Imaging**
  HCR™ RNA-FISH enables analog relative quantitation of RNA and/or protein targets with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos) (Trivedi et al., 2018, Choi et al., 2018, Schwarzkopf et al., 2021).
- **Single-Molecule Quantitative RNA Imaging**
  HCR™ RNA-FISH enables digital RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016, Choi et al., 2018).
- **Read-Out/Read-In Analysis Framework**
  The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Protocols in Diverse Sample Types**
  Protocols for HCR™ RNA-FISH and/or IF in diverse sample types are adapted from the zoo paper (Choi et al., 2016):
  - bacteria in suspension
  - FFPE human tissue sections
  - generic sample in solution
  - generic sample on a slide
  - mammalian cells on a slide
  - mammalian cells in suspension
  - whole-mount chicken embryos
  - whole-mount fruit fly embryos
  - whole-mount mouse embryos
  - whole-mount nematode larvae
  - whole-mount sea urchin embryos
  - whole-mount zebrafish embryos and larvae
- **HCR™ RNA Flow Cytometry**
  HCR™ RNA Flow Cytometry enables analog RNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria without the need to engineer reporter lines (Choi et al., 2018).
- **HCR™ Northern Blots**
  HCR™ Northern Blots enable simultaneous quantification of RNA target size and abundance with automatic background suppression throughout the protocol (Schwarzkopf & Pierce, 2016).
- **HCR™ Amplifiers**
  HCR™ Amplifiers enable multiplex, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).